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Prevalence of β -lactamase-producing bacteria in human periodontitis

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Background and Objective: Beta-lactam antibiotics prescribed in periodontal therapy are vulnerable to degradation by bacterial β -lactamases. This study evaluated the occurrence of β -lactamase-positive subgingival bacteria in chronic periodontitis subjects of USA origin, and assessed their *in vitro* resistance to metronidazole at a breakpoint concentration of 4 μ g/mL.

Material and Methods: Subgingival plaque specimens from deep periodontal pockets with bleeding on probing were removed from 564 adults with severe chronic periodontitis before treatment. The samples were transported in VMGA III and then plated onto: (i) nonselective enriched Brucella blood agar (EBBA) and incubated anaerobically for 7 d; and (ii) selective trypticase soy-bacitracin-vancomycin (TSBV) and incubated for 3 d in air + 5% CO₂. At the end of the incubation periods, the bacterial test species were identified and quantified. Specimen dilutions were also plated onto EBBA plates supplemented with 2 μ g/mL of amoxicillin, a combination of 2 μ g/mL of amoxicillin plus 2 μ g/mL of the β -lactamase inhibitor clavulanic acid, or 4 μ g/mL of metronidazole, followed by anaerobic incubation for 7 d. Bacterial test species presumptively positive for β -lactamase production were identified by growth on EBBA primary isolation plates supplemented with amoxicillin alone and no growth on EBBA primary isolation plates containing both amoxicillin plus clavulanic acid. A subset of such isolates was subjected to nitrocefin-based chromogenic disk testing to confirm the presence of β -lactamase activity. *In vitro* resistance to 4 μ g/mL of metronidazole was noted when growth of test species occurred on metronidazole-supplemented EBBA culture plates.

Results: Two-hundred and ninety-four (52.1%) of the study subjects yielded β -lactamase-producing subgingival bacterial test species, with *Prevotella intermedia/nigrescens*, *Fusobacterium nucleatum* and other *Prevotella* species most frequently identified as β -lactamase-producing organisms. Of the β -lactamase-producing bacterial test species strains recovered, 98.9% were susceptible *in vitro* to metronidazole at 4 μ g/mL.

Conclusion: The occurrence of β -lactamase-positive subgingival bacterial species in more than half of the subjects with severe chronic periodontitis raises questions about the therapeutic potential of single-drug regimens with β -lactam antibiotics in periodontal therapy. The *in vitro* effectiveness of metronidazole against nearly all recovered β -lactamase-producing subgingival bacterial species further supports clinical periodontitis treatment strategies involving the combination of systemic amoxicillin plus metronidazole.

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Beta-lactamase production represents a major virulence factor by which pathogenic bacteria evade the broad-spectrum antimicrobial effects of β -lactam antibiotics and perpetuate human infections, including those in orofacial tissues (1). Bacterial β -lactamases rapidly hydrolyze amide bonds within the four-membered ring forming the foundational structure of β -lactam antibiotics, leaving them pharmacologically inactive as antimicrobial agents that disrupt bacterial cell-wall peptidoglycan biosynthesis (1).

Beta-lactamase activity has been detected in subgingival sites of subjects with chronic periodontitis at levels capable of inactivating β -lactam antibiotics passing into periodontal pockets through gingival crevicular fluid exudate (2). Subgingival β -lactamase has been significantly correlated with increasing periodontal probing depth measurements (2), recent treatment with systemic penicillin drugs (3), and carriage of β -lactamase-encoding genes by microbial species in subgingival plaque biofilms (4). Studies of patients with chronic periodontitis in the USA (3–5), The Netherlands (6,7), Spain (7), Norway (8), France (9,10) and the UK (11) have reported a 53.2–100% occurrence in subjects for subgingival β -lactamase-producing bacteria, with higher prevalence rates found in localities with greater over-the-counter access and consumption of systemic antimicrobial agents (7). However, these findings are limited by their inclusion of relatively few study subjects (12–47 patients with periodontitis per study), who were mostly dental-school patients and/or from localized geographic regions that may not necessarily be representative of more diverse community populations. For example, data from the USA on the occurrence of subgingival β -lactamase-producing bacterial species in patients with chronic periodontitis is presently derived from a total of 42 dental-school patients in Connecticut and 25 in Florida (3,5).

As a result, there is a need to assess subgingival β -lactamase-producing bacteria in larger-sized subject groups that are geographically distributed beyond a single city or dental-school patient population. The aim of the present study was to evaluate the occurrence of β -lactamase-positive subgingival bacteria in 564 geographically distributed subjects in the USA with chronic periodontitis and to assess their *in vitro* resistance to metronidazole at a breakpoint concentration of 4 μ g/mL.

Material and methods

Subjects

A total of 564 adults (270 men and 294 women; age range, 33–91 years; mean age \pm standard deviation = 49.1 ± 11.7 years), diagnosed with severe chronic periodontitis (12) by periodontists in private dental practices in the USA, were included in the present study as their subgingival plaque samples were consecutively received for microbiological analysis by the Oral Microbiology Testing Service (OMTS) Laboratory at Temple University School of Dentistry, Philadelphia (PA, USA). Three-hundred and fifty-four (62.8%) of the study subjects originated from Maryland ($n = 174$), Pennsylvania ($n = 91$), New Jersey ($n = 51$), Delaware ($n = 25$), Virginia ($n = 7$) and the District of Columbia ($n = 6$) in the mid-Atlantic region of the USA, with all others from Connecticut ($n = 48$), Florida ($n = 35$), Illinois ($n = 20$), 11 other states in the eastern USA ($n = 47$) and Texas ($n = 60$). Persons identified with aggressive periodontitis, or with antibiotic use in the past 6 mo, were excluded. Approval for the study was provided by the Temple University Human Subjects Protections Institutional Review Board.

Microbial sampling and transport

Subgingival plaque specimens were obtained by the diagnosing periodon-

tists, who followed a standardized sampling protocol, before treatment from three to five deep (≥ 6 mm) periodontal pockets in each subject that exhibited bleeding on probing during the initial diagnostic evaluation. After isolation with cotton rolls, and removal of saliva and supragingival deposits, one or two sterile, absorbent, paper points (Johnson & Johnson, East Windsor, NJ, USA) were advanced into each selected periodontal site for approximately 10 s. Upon removal, all paper points per study subject were pooled in a glass vial containing six to eight small glass beads and 2.0 mL of anaerobically prepared and stored VMGA III transport medium (13), which possesses a high preservation capability for oral microorganisms during postsampling transit to the laboratory (13,14). The subgingival samples were then transported within 24 h to the OMTS Laboratory, which is licensed for high-complexity bacteriological analysis by the Pennsylvania Department of Health. The OMTS Laboratory is also federally certified by the United States Department of Health and Human Services to be in compliance with Clinical Laboratory Improvement Amendments-mandated proficiency testing, quality control, patient test management, personnel requirements and quality assurance standards required of clinical laboratories engaged in diagnostic testing of human specimens in the USA (15). All laboratory procedures were performed by personnel who were blinded to the clinical status of the study subjects and their inclusion in the present analysis.

Microbial culture and incubation

At the OMTS Laboratory, the specimen vials were warmed to 35°C to liquefy the VMGA III transport medium, and sampled microorganisms were mechanically dispersed from the paper points with a Vortex mixer, which was used at the maximal setting for 45 s.

Serial, 10-fold dilutions of the dispersed bacteria were prepared in Möller's VMG I anaerobic dispersion solution, comprised of prerduced, anaerobically sterilized, 0.25% tryptose, 0.25% thiotone E peptone and 0.5% NaCl (13). Then, 0.1-mL dilution aliquots were spread, with a sterile bent glass rod, onto nonselective enriched Brucella blood agar (EBBA) primary isolation plates (16), composed of 4.3% Brucella agar supplemented with 0.3% bacto-agar, 5% defibrinated sheep blood, 0.2% hemolyzed sheep red blood cells, 0.0005% hemin and 0.0005% menadione, and onto selective trypticase soy–bacitracin–vancomycin (TSBV) agar (17). The inoculated EBBA plates were incubated at 35°C for 7 d in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI, USA) containing 85% N₂, 10% H₂ and 5% CO₂, and the TSBV plates were incubated at 35°C for 3 d in air + 5% CO₂.

Test species identification

Total anaerobic viable counts, and counts of the test species *Porphyromonas gingivalis*, *Prevotella intermedia/nigrescens*, other *Prevotella* species (including *Prevotella melaninogenica* and nonpigmented *Prevotella* species), *Fusobacterium nucleatum*, *Parvimonas micra*, *Capnocytophaga* species, *Streptococcus constellatus*, *Centipeda periodontii*, *Enterococcus faecalis* and staphylococci, were made on nonselective EBBA primary isolation plates using a ring-light magnifying loupe, presumptive phenotypic methods previously described (18–21) and the RapID ANA II (Innovative Diagnostic Systems, Atlanta, GA, USA) micro-method kit system for selected isolates. *Aggregatibacter actinomycetemcomitans*, gram-negative enteric rods/pseudomonads and *Candida* species were quantitated on selective TSBV agar, as previously described (16,17). The proportional recovery of each test species was ascertained in each subject by calculating the percentage of test species colony-forming units relative to total subgingival anaerobic viable counts, as determined on nonselective EBBA primary isolation plates.

Detection of β -lactamase-producing organisms

Additional 0.1 mL aliquots of subgingival sample dilutions were inoculated onto EBBA primary isolation plates supplemented with either 2 μ g/mL of amoxicillin, which was previously established as a susceptibility breakpoint for aminopenicillin antibiotics (22), or a combination of 2 μ g/mL of amoxicillin plus 2 μ g/mL of the β -lactamase-inhibitor, clavulanic acid (23), followed by anaerobic incubation. Direct colony suspensions (equivalent to a 0.5 McFarland standard) of pure *A. actinomycetemcomitans* isolates from selective TSBV were subcultured onto these media as their identification is frequently obscured within mixed bacterial populations on nonselective EBBA primary isolation plates (17).

A surface-overlay technique was used to add clavulanic acid, at a concentration of 2 μ g/mL, to amoxicillin-containing EBBA primary isolation plates (24). Using a sterile glass rod, 0.1 mL of a fresh 400- μ g/mL solution of clavulanic acid, prepared by dissolving 4.2 mg of a 95.3% pure lithium clavulanate powder (provided by SmithKline Beecham, Collegeville, PA, USA) into 10 mL of sterile 0.1 M phosphate buffer (pH 6.0), was evenly spread over the surfaces of EBBA plates supplemented with 20 mL of amoxicillin. The plates were held at room temperature for 30 min to allow surface drying and subsurface drug diffusion, and then inoculated with subgingival plaque specimens.

Test species positive for β -lactamase production were presumptively identified by growth on EBBA primary isolation plates supplemented with amoxicillin alone and by no growth on EBBA primary isolation plates containing both amoxicillin and clavulanic acid (6,7). A subset of 50 such presumptively identified isolates was subjected to a nitrocefin-based qualitative chromogenic disk assay (BBL Cefinase; BD Diagnostic Systems, Sparks, MD, USA), following the manufacturer's instructions, to confirm the presence of β -lactamase activity.

In vitro antibiotic-resistance testing

Aliquots (0.1 mL) of subgingival sample dilutions were also inoculated onto EBBA primary isolation plates supplemented with metronidazole at a susceptibility breakpoint concentration of 4 μ g/mL (22), and incubated anaerobically for 7 d, in order to assess *in vitro* resistance to metronidazole among β -lactamase-producing clinical isolates. *In vitro* resistance to metronidazole was defined as test species growth on metronidazole-supplemented EBBA primary isolation plates (16,20,25,26). *Bacteroides thetaiotaomicron* ATCC 29741, *Clostridium perfringens* ATCC 13124 and a multiantibiotic-resistant clinical periodontal isolate of *F. nucleatum* were employed as positive and negative quality controls for the antibiotic resistance testing. All antimicrobials were obtained as pure powder from Sigma-Aldrich (St Louis, MO, USA).

Data analysis

Descriptive analyses were used to calculate mean subject age and standard deviation values, the occurrence and proportional cultivable recovery of test species in subjects, the occurrence and proportional recovery of β -lactamase-producing organisms in subjects, and the occurrence in subjects of *in vitro* metronidazole drug resistance among the subgingival clinical isolates. Data analysis was performed using the SAS 9.2 for Windows (SAS Institute, Inc., Cary, NC, USA) statistical software package.

Results

A subset of 50 test species, presumptively identified as β -lactamase positive by their growth on amoxicillin-supplemented EBBA primary isolation plates and by no growth on amoxicillin plus clavulanic acid-supplemented EBBA primary isolation plates, were all confirmed as β -lactamase-producing organisms using the nitrocefin chromogenic disk assay (data not shown).

A total of 294 (52.1%) study subjects yielded β -lactamase-producing subgingival test species. *P. intermedia*/

nigrescens, *F. nucleatum* and other *Prevotella* species were most frequently identified as β -lactamase-positive species, with 51.0% of all study subjects with cultivable *P. intermedia/nigrescens*, 24.6% with *F. nucleatum* and 66.2% with other *Prevotella* species exhibiting β -lactamase-producing strains of these species (Table 1). Beta-lactamase production was also found among 0.8% of *P. micra* species, 2.0% of *Capnocytophaga* species and 5.0% of gram-negative enteric rods/pseudomonads in culture-positive subjects (Table 1).

A single β -lactamase-producing subgingival test species was recovered from 236 (80.3%) subjects, whereas 54 (18.4%) and four (1.3%) subjects each yielded two and three different β -lactamase-producing test species, respectively (Fig. 1).

Subgingival proportions of β -lactamase-positive *P. intermedia/nigrescens*, *F. nucleatum* and *P. micra* averaged 6.7–9.9%, whereas mean subgingival recovery levels of <1% were found for β -lactamase-positive isolates of other *Prevotella* species and for *Capnocytophaga* species (Table 1). Three-hundred and fifty-three (98.9%) of the 357 β -lactamase-

producing subject test species recovered (Table 1) were susceptible *in vitro* to 4 μ g/mL of metronidazole, except for one *P. intermedia/nigrescens* strain, one *F. nucleatum* strain and gram-negative enteric rods/pseudomonads in two subjects.

All recovered strains of *P. gingivalis*, *A. actinomycetemcomitans*, *S. constellatus*, *C. periodontii*, *E. faecalis*, and *Staphylococcus* species, failed to demonstrate presumptive β -lactamase activity (Table 1).

Discussion

These findings confirm and extend previous studies revealing subgingival β -lactamase-producing bacteria to be present in a majority of patients with chronic periodontitis (3–11). A strength of the present study is that it assessed the largest group, to date, of subjects with chronic periodontitis for subgingival β -lactamase-producing bacteria ($n = 564$); similar, previous, investigations have collectively examined a total of only 255 subjects with chronic periodontitis (3,5–11). The present study data also evaluated a private dental practice-based convenience sample of subjects with chronic peri-

odontitis originating from a wider geographic region than in previous reports from the USA on subgingival β -lactamase activity (2,3,5), which may permit the findings to be more generalizable to a broader range of USA community population groups.

Owing to the instability of clavulanic acid in agar-dilution plates stored for more than 3 d (24), a surface-overlay technique was used in this study to add clavulanic acid to amoxicillin-containing EBBA primary isolation plates immediately before *in vitro* testing. As clavulanic acid binds irreversibly to β -lactamases and prevents inactivation of β -lactam antibiotics (23), microbial growth patterns on these plates were compared with those on EBBA plates supplemented with amoxicillin only as a presumptive method for detecting β -lactamase-positive subgingival bacterial species. This approach was validated with confirmatory nitrocefin chromogenic disk testing for β -lactamase activity from presumptively detected subgingival bacterial species in previous studies (6,7), as well as on a subset of such subject isolates in the present study.

In this study, 52.1% of subjects with untreated chronic periodontitis

Table 1. Occurrence and proportional subgingival recovery of β -lactamase-positive test species in 564 adults with severe chronic periodontitis

Species	Culture-positive subjects n (%) ^b	Subjects with β -lactamase-positive isolates ^c n (%) ^b (%) ^d	Recovery of β -lactamase-positive isolates (mean percentage \pm standard error)	Range %
<i>Prevotella intermedia/nigrescens</i>	449 (79.6)	229 (40.6/51.0)	9.6 \pm 0.8	0.001–62.1
<i>Fusobacterium nucleatum</i>	293 (52.0)	72 (12.8/24.6)	6.7 \pm 0.9	0.001–29.4
Other <i>Prevotella</i> species ^a	68 (12.1)	45 (8.0/66.2)	0.6 \pm 0.5	0.001–24.2
<i>Capnocytophaga</i> species	251 (44.5)	5 (0.9/2.0)	0.3 \pm 0.1	0.002–0.6
<i>Parvimonas micra</i>	495 (87.8)	4 (0.7/0.8)	9.9 \pm 3.9	1.5–19.7
Enteric rods/pseudomonads	40 (7.1)	2 (0.4/5.0)	1.7 \pm 0.8	0.9–2.5
<i>Porphyromonas gingivalis</i>	197 (34.9)	0	0	0
<i>Aggregatibacter actinomycetemcomitans</i>	85 (15.1)	0	0	0
<i>Streptococcus constellatus</i>	267 (47.3)	0	0	0
<i>Centipeda periodontii</i>	4 (0.7)	0	0	0
<i>Enterococcus faecalis</i>	5 (0.9)	0	0	0
<i>Staphylococcus</i> species	4 (0.7)	0	0	0

^aIncludes *Prevotella melaninogenica* and nonpigmented *Prevotella* species.

^bAmong all study subjects.

^cSpecies growth on amoxicillin-supplemented enriched Brucella blood agar (EBBA) primary isolation plates, with no growth on similar plates with a clavulanic acid overlay.

^dAmong species-positive study subjects.

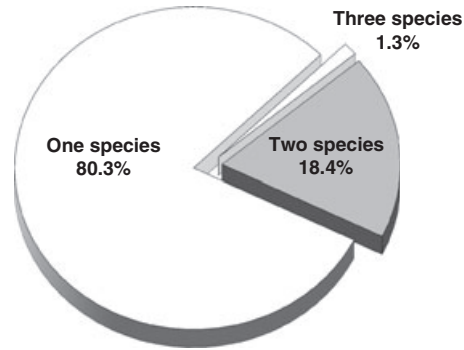


Fig. 1. Occurrence of one or more periodontal bacterial pathogens producing β -lactamase among 294 β -lactamase-positive subjects with chronic periodontitis.

yielded β -lactamase-producing subgingival bacteria, which is at the lower end of the prevalence rates of 53.2–100% reported in previous studies (3,5–11). This may be because of differences in periodontal disease severity, treatment status and recent systemic antibiotic usage among the evaluated subjects with chronic periodontitis and the microbiological methods employed. For example, some previous studies evaluated only subjects with 'refractory' chronic periodontitis, responding poorly to conventional mechanical periodontal therapy (5,8). As systemic antimicrobial therapy is frequently employed in such patients (27), which is positively correlated with increased subgingival β -lactamase activity (3), it is not surprising that a greater occurrence of β -lactamase-producing subgingival bacteria would be reported for patients with 'refractory' periodontitis compared with patients with untreated chronic periodontitis and without antibiotic use within the past 6 mo, as in the present study. In this regard, the 52.1% occurrence of subgingival β -lactamase-producing bacteria in the present study is similar to the 48% rate documented among 21 patients with chronic periodontitis not exposed to antibiotic therapy over the previous 12 mo (3). Moreover, a higher occurrence of subgingival β -lactamase would also be expected among subjects with chronic periodontitis from countries, such as in southern Europe, with greater antibiotic over-the-counter access and consumption rates than found in the USA (28).

P. intermedia/nigrescens, *F. nucleatum* and other *Prevotella* species were the most frequently identified β -lactamase-positive species in the present study, consistent with previous investigations (3,5–10). Subgingival recovery of cultivable enzyme-producing strains of *P. intermedia/nigrescens* and *F. nucleatum* averaged 9.6% and 6.7%, respectively, of total anaerobic viable counts in β -lactamase-positive subjects, whereas other *Prevotella* species occurred in lower proportions, averaging <1%. *P. micra*, *Capnocytophaga* species and gram-negative enteric rods/pseudomonads were also found to be β -lactamase positive in some study subjects. Interestingly, all 197 strains of *P. gingivalis* found in subjects in the present study were β -lactamase negative, in agreement with most previous studies of subgingival isolates (6,9,29). In contrast, 7.7% of 26 subgingival *P. gingivalis* strains were reported to be β -lactamase-positive in one study (30), and 25.5% of 51 strains in another study (31) were found *in vitro* to be resistant to amoxicillin, but susceptible to amoxicillin/clavulanic acid, indicative of β -lactamase production. Additional β -lactamase-positive microbial species and subjects would probably be identified using more sensitive methods, such as molecular analysis of bacterial 16S ribosomal RNA gene sequences (5,8) or β -lactamase-encoding genes (4,10,32,33), than the culture-based procedures employed in the present study.

This study also did not classify β -lactamase phenotypes, test for

β -lactamase groups resistant to clavulanic acid or quantitate the strength of subgingival β -lactamase activity present in β -lactamase-positive subjects (1,2). Additionally, the private practice periodontists who diagnosed the study subjects were not calibrated in their assessments, although support for their diagnosis of severe chronic periodontitis was evidenced by their identification for microbiological sampling of three or more periodontal sites per subject with probing depths of ≥ 6 mm, which strongly correlates (94.1% positive predictive value) with the presence of severe periodontal attachment loss in adults (34).

The presence of β -lactamase activity in periodontal pockets poses potentially important therapeutic implications relative to the control of mixed populations of subgingival periodontal bacterial pathogens in dental plaque biofilms. Enzymatic degradation of β -lactam antibiotics may permit subgingival perpetuation of β -lactamase-producing microbial species and other organisms in the subgingival microbiota that otherwise would be suppressed by β -lactam antibiotics (1). An increasing prevalence of β -lactamase activity among oral bacterial isolates has been reported (35), which is largely thought to be the result of plasmid and transposon-mediated conjugal transfer and dissemination of β -lactamase-encoding genes among microorganisms surviving following selective pressure from β -lactam antibiotics (1). Beta-lactamases may predispose systemic penicillin monotherapy, unprotected by β -lactamase inhibitors, to oral cavity treatment failures, such as recalcitrant orofacial infections (36,37) and periodontal surgical flap necrosis (38), as well as enhance periodontal abscess risk in untreated periodontitis subjects (39,40).

Penicillin and amoxicillin are among the systemic antibiotics most frequently prescribed by periodontists (41). However, relatively little research attention has been given to their adjunctive use in periodontal therapy in the absence of β -lactamase inhibitors (42,43), with systemic phenoxymethyl penicillin failing to provide

significant treatment benefits in a clinical trial of patients with aggressive periodontitis (44). It is noteworthy that subgingival bacterial isolates resistant to 2 µg/mL of amoxicillin were found to show a transient increase from a pretreatment baseline of 0.5% to 35% (a 70-fold increase), over the course of an adjunctive 14-d systemic amoxicillin drug regimen, in patients with chronic periodontitis (45). In that study, *P. intermedia/nigrescens* and *P. melaninogenica*, often identified as β-lactamase-producing species in the present and previous studies (3,5–10), were found to be among the most prevalent amoxicillin-resistant organisms (45). The extent to which bacterial β-lactamase activity contributed to these *in vivo* microbiological shifts was not addressed in the study and remains to be delineated in future investigations.

Finally, the present study found 98.9% of recovered subgingival β-lactamase-producing bacteria to be susceptible *in vitro* to 4 µg/mL of metronidazole, an observation consistent with previous reports (3,5,8). As a result, metronidazole suppression of β-lactamase-positive microbial species may, in part, help to protect concurrently administered amoxicillin from *in vivo* degradation by bacterial β-lactamases, better enabling pharmacologically active amoxicillin to reach penicillin-binding proteins on bacterial cell membranes to exert antimicrobial effects (1), and contributing to the documented clinical and microbiological benefits of systemic amoxicillin plus metronidazole in periodontal therapy (46–49). However, this may not occur in patients from certain geographic regions, such as Columbia, where marked *in vitro* metronidazole resistance may be found among β-lactamase-positive subgingival isolates of *P. gingivalis*, *P. intermedia/nigrescens*, *P. melaninogenica* and *F. nucleatum* (31).

In conclusion, the occurrence of β-lactamase-positive subgingival bacterial species in more than one-half of subjects with severe chronic periodontitis raises questions about the therapeutic potential of single-drug regimens with β-lactam anti-

biotics in periodontal therapy. The *in vitro* effectiveness of metronidazole against nearly all recovered β-lactamase-producing subgingival bacterial species further supports clinical periodontitis treatment strategies involving the combination of systemic amoxicillin plus metronidazole.

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